1. Title:
Host range and molecular diagnostics of *Fusarium* wilt of birdsfoot trefoil

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4. Abstract:
A vascular wilt of birdsfoot trefoil (*Lotus corniculatus*) caused by *Fusarium oxysporum* was first reported in the 1970s in trefoil seed production fields in the Champlain Valley of New York and Vermont and in the late 1980s in trefoil forage production fields in western New York, and the disease continues to be a problem in both regions. Infected plants display root necrosis and wilt as temperatures increase in the summer, leading to high rates of mortality in the seeding year. However, accurate diagnosis of the disease has always been problematic. The symptoms – wilting, yellowing of the foliage, root necrosis, and plant death – can also be caused by other factors, both biotic and abiotic, and the causal fungus is morphologically indistinguishable from the ubiquitous nonpathogenic *F. oxysporum* routinely isolated from roots. Accurate diagnosis requires isolating *F. oxysporum* from the symptomatic plant, inoculating a healthy trefoil plant with the isolated fungus, and waiting to see if wilt symptoms develop, a process that is costly in both time and resources. Management of the pathogen has been further complicated by a lack of understanding of its host range. Most pathogenic strains of *F. oxysporum* cause symptoms on multiple host plants, and it was unknown whether the trefoil-wilt *F. oxysporum* caused symptoms, with corresponding yield loss, on other legumes commonly grown by New York growers. Five isolates of *F. oxysporum* pathogenic to trefoil and three isolates of *F. oxysporum* pathogenic to other hosts were used to inoculate alfalfa, red clover, trefoil, soybeans, dry beans, and peas in a replicated greenhouse experiment. Cultivars with known *F. oxysporum* susceptibility were used, and inoculations followed standardized procedures developed by previous researchers. The trefoil-wilt *F. oxysporum* caused severe wilting and root necrosis of trefoil, moderate wilting and root necrosis of peas, and mild root necrosis of red clover, but it did not cause symptom development on alfalfa, soybeans or dry beans. The results suggest that peas and red clover may suffer light to moderate yield losses when planted in infested fields, but that alfalfa, dry beans and soybeans are likely to be unaffected. To evaluate the feasibility of developing a DNA-based diagnostic procedure, the complete intergenic spacer region and portions of two other genes were sequenced for 28 isolates of the trefoil-wilt *F. oxysporum*, and the sequences were compared to corresponding sequences from *F. oxysporum* nonpathogenic to trefoil, including *F. oxysporum* pathogenic to other hosts. The results indicate that the trefoil-wilt *F. oxysporum* populations found in New York and Vermont production fields are very closely related to each other but genetically distinct from other *F. oxysporum*, suggesting that it will be possible to develop a single DNA-based polymerase chain reaction diagnostic test for the pathogen. Development of the diagnostic test will commence in early 2007, and the test should be completed within a year.

5. Background and Justification:

Since the 1970s, production of birdsfoot trefoil in New York and Vermont has been limited by a vascular wilt caused by *Fusarium oxysporum*. Infected plants display root necrosis and wilt as temperatures increase in the summer, leading to high rates of mortality in the seeding year (figures 1, 2 and 3). The wilt was first reported in the 1970s in trefoil seed production fields in the Champlain Valley of New York and Vermont[1,2] and in the late 1980s in trefoil forage production fields in western New York.[3] Survey work and pathogenicity testing conducted in 2004 and 2005 confirmed that the wilt is still present in trefoil production fields in both regions.[4] Most trefoil cultivars, including Bull, Dawn, Empire, Leo and Viking, are highly susceptible to the wilt pathogen;[5] only Pardee, a cultivar recently developed by researchers at Cornell, demonstrates a moderate level of resistance.

However, the effectiveness of ongoing efforts to breed trefoil for resistance to the pathogen has been unclear. Pardee was developed by screening germplasm against a single isolate of the pathogen collected in a Western New York production field in the mid-1990s, and it was unknown whether that isolate was representative of the entire population of the pathogen. Many *formae speciales* of *F. oxysporum* are polyphyletic,[6] encompassing multiple lineages with different evolutionary origins and different pathogenic properties, and resistance to isolates from one evolutionary lineage does not necessarily confer resistance to isolates from other lineages. Characterization of the pathogen population would help ensure that trefoil bred for resistance to the pathogen exhibits resistance in all regions where the pathogen is found.

Management of the *Fusarium* wilt has also been limited by a lack of understanding of the host range of the pathogen. Wilts caused by *F. oxysporum* are classified into *formae speciales*, or strains, by the range of hosts that develop wilt symptoms upon infection by that *F. oxysporum*, and many *formae speciales* cause rapid wilting and plant death of multiple hosts.[7] Individual *formae speciales* also cause root necrosis, with corresponding yield loss, of many additional hosts, with the range of hosts affected differing by *forma specialis*. Prior to this study, the host range of the *F. oxysporum* causing rapid wilt of birdsfoot trefoil remained poorly investigated, and the effect of the pathogen on common rotational crops with known susceptibility to *F. oxysporum* was unknown. An improved understanding of the host range would help minimize economic losses caused by the pathogen by identifying crops that can be planted, with minimal expected yield loss, in infested fields.

Management of the pathogen has been further hampered by inadequate diagnostic tools. Because morphological differences among *formae speciales* of *F. oxysporum* and between pathogenic and non-pathogenic strains of *F. oxysporum* are not apparent, accurate diagnosis of the pathogen requires pathogenicity testing. After isolation of the pathogen from symptomatic plants and establishment of cultures on artificial media, trefoil plants must be inoculated and monitored for symptom development. The procedure is costly in time, resources, and facilities and, due to the tendency of *F. oxysporum* to lose its pathogenicity in artificial culture, is inherently unreliable. As a consequence, extensive surveys for the pathogen have not been conducted, and the full distribution of the wilt *Fusarium* remains unclear. The development of diagnostic PCR primers would increase the speed and accuracy of pathogen diagnosis while greatly reducing the associated costs.

6. Objectives:

(1) Evaluate the host range of isolates of *F. oxysporum* causing rapid wilt on trefoil

(2) Develop PCR-based diagnostic primers for rapid identification of the *F. oxysporum* causing rapid wilt on trefoil
(3) Project evaluation

7. Procedures:

Objective 1: Evaluate the host range of isolates of *F. oxysporum* causing rapid wilt on trefoil

A. Greenhouse assay

Beans (*Phaseolus vulgaris*), soybeans (*Glycine max*), peas (*Pisum sativum*), alfalfa (*Medicago sativa*), red clover (*Trifolium pretense*), and birdsfoot trefoil (*Lotus corniculatus*) were screened for their susceptibility to the trefoil wilt *Fusarium*. Inoculations were conducted with eight isolates of *F. oxysporum* and a negative control. Five isolates of the trefoil wilt *Fusarium* were tested, including two isolates from Vermont and three from New York. Both historical (collected in the early to mid 1990s) and contemporary (collected in 2004) isolates were included. *F. oxysporum* isolates with documented virulence against peas, alfalfa and clover were included as positive controls; sterile Czapek-Dox broth, the media used for growing the *F. oxysporum* cultures, was used as a negative control. Isolates with known virulence against beans and soybeans were not included as positive controls because lack of availability of the corresponding cultures. Host cultivars with documented susceptibility to *F. oxysporum* were selected: *P. vulgaris* cv. U.I. 114,[8, 9] *G. max* cv. Essex,[10,11] *P. sativum* cv. M410,[12] *M. sativa* cv. MNGN-1,[13] *T. pratense* cv. Chesapeake,[14] and *L. corniculatus* cv. Georgia-1.[5] Individual plants were grown in SC10 Super Cell Cone-tainers (Stuewe and Sons, Inc; Corvallis, OR). In each experiment, 12 plants of each host were inoculated with each isolate. A randomized block design was used, and all plants were maintained in the greenhouse. The experiment was repeated three times; a total of 36 plants were tested for each host-isolate combination. Trefoil, alfalfa, clover, bean, pea, and soybean were inoculated with spore suspensions ($1x10^6$, spores/ml) 10 weeks, 8 weeks, 6 weeks, 10 days, 10 days, and 8 days after seeding, respectively. Trefoil, alfalfa and clover roots were soaked in spore suspensions for 30 minutes; bean and soybean roots, for 5 minutes; and pea roots, for 1-2 minutes. For alfalfa, clover, beans, soybeans, and peas, all soil was shaken free of the roots and the lowest fifth of the roots was removed prior to soaking. For trefoil, the root mass was cut 6 to 8 cm below the crown, and the entire root mass (with it associated soil) was soaked. Trefoil, alfalfa, clover, beans and soybeans were monitored for wilt symptoms for 65 days after inoculation. Peas were monitored for 45 days. Wilted stems were collected and surface sterilized, and segments of the stems were plated onto eighth-strength PDA. If cultures representative of *F. oxysporum* grew from the ends of the stem segments (figure 4), the cultures were transferred to an artificial media selective for *Fusarium* sp. to confirm culture identity. A plant was considered positive for *Fusarium* wilt only if the cultures isolated from the ends of the stem segments displayed growth characteristics typical of *F. oxysporum* on the selective growth media. At the end of the experiment, plants were uprooted and their roots split longitudinally. Root necrosis was estimated visually and recorded on a 0 to 5 scale. An isolate was only considered virulent on a particular host if it caused vascular wilt of one or more plants in at least two of the three repeated experiments.

B. Sequence analysis

Because evaluating the host range of the trefoil wilt pathogen in the greenhouse is expensive and time-consuming, the host range of the pathogen was indirectly evaluated for other hosts using sequence data. Because *F. oxysporum* exhibits only asexual reproduction, evolutionary lineages within *formae speciales* of *F. oxysporum* are highly clonal. If the *F. oxysporum* causing

trefoil wilt is actually a previously described *forma specialis* with a broader host range than formerly recognized, it would be expected to share exact sequence identity with isolates from at least one lineage of that *forma specialis*. Portions of the elongation factor (EF-1_) and mitochondrial small subunit ribosomal DNA (mt SSU rDNA) genes were sequenced, as well as the complete nuclear ribosomal intergenic spacer (IGS) region (~2.6 kb). Twenty-eight isolates of the trefoil wilt pathogen, collected from New York and Vermont between the mid-1980s and 2004, three *F. oxysporum* isolates isolated from trefoil roots in 2004 but not pathogenic to trefoil, and eight *F. oxysporum* isolates pathogenic to other hosts were sequenced. Sequence data were compared to published sequences of other *formae speciales* of *F. oxysporum* obtained from GenBank. Phylogenetic analyses will be conducted on the sequence data to illustrate the hypothesized evolutionary relationship of the trefoil-wilt *F. oxysporum* to other *formae speciales* of *F. oxysporum.*

Objective 2: Develop PCR-based diagnostic primers for rapid identification of the *F. oxysporum* causing rapid wilt on trefoil

The sequencing of genes of the *F. oxysporum* isolates required more time than anticipated, and diagnostic primer development will not commence until February. However, the sequence data indicate that the trefoil-wilt *F. oxysporum* is a clonal lineage distinct from other *F. oxysporum*, and development of a single set of diagnostic primers specific to the trefoil wilt pathogen should be possible. Nested PCR primers will be developed, with the first set of primers specific to *F. oxysporum* and an internal set of primers specific to the trefoil wilt *F. oxysporum.* The development of trefoil-wilt-specific PCR primers will be attempted using known sequence polymorphisms within the IGS region, with the outer *F. oxysporum*-specific primers based on sequence polymorphisms within the IGS region and/or the surrounding 28S and 16S nuclear rDNA genes. If development of trefoil-wilt-specific primers is not possible within the IGS region, the primers will be developed from the sequence of a unique random amplified polymorphic DNA (RAPD) amplification product specific to the trefoil-wilt pathogen. Primer development should be completed by April or May 2008.

Objective 3: Project evaluation

A. Grower feedback

Because sequence analyses were not completed until December 2006 and primer development has yet to commence, efforts to contact growers affected by the pathogen have been postponed. At the conclusion of all laboratory work, results will be individually communicated to growers in the Champlain valley and in western New York with fields known to be affected by the pathogen as well as extension personnel in both states.

B. Primer specificity and sensitivity

Upon completion of primer development, the specificity of the diagnostic primers will be tested using DNA of *F. oxysporum* isolates pathogenic and nonpathogenic to trefoil. The specificity of the primers will be further tested on symptomatic trefoil plants (grown in soil collected from a field in western New York known to have problems with the trefoil-wilt Fusarium) and non-symptomatic plants (grown in sterilized greenhouse soil). The sensitivity of the primers will be tested using serial dilutions of trefoil-wilt *F. oxysporum* DNA.

8. Results and discussion:

The trefoil-wilt *F. oxysporum* causes moderate vascular wilt and root necrosis of peas and mild root necrosis of red clover but does not appear to be pathogenic to alfalfa, beans, or soybeans (tables 1 and 2). These results suggest that the trefoil pathogen may cause moderate

yield losses in peas and may also negatively impact yields of red clover. Alfalfa, beans and soybeans should be unaffected by the pathogen and can likely be safely planted in infested fields.

Analysis of the complete IGS region and portions of the EF-1_ and mtSSU rDNA genes suggest that the trefoil-wilt *F. oxysporum* populations in New York and Vermont share a common evolutionary origin. Sequence data were identical for all 28 isolates of the pathogen tested, regardless of sampling location or year. The genetic similarity of the isolates suggests that the pathogen should exhibit comparable biological characteristics, including aggressiveness and virulence, throughout its known distribution. This conclusion is supported by the results from the host range experiment, in which all five trefoil-wilt isolates tested produced highly similar wilt and necrosis responses on the six host plants tested (tables 1 and 2). The results indicate that the use of a single isolate for screening trefoil germplasm in ongoing breeding efforts should lead to trefoil broadly adapted to all regions where the wilt pathogen is present.

The multilocus DNA sequence typing of the 28 isolates pathogenic to trefoil, 3 isolates nonpathogenic to trefoil and 8 isolates pathogenic to other hosts, and the subsequent comparison of the sequences with previously published sequence data for other *F. oxysporum* isolates indicates that the trefoil-wilt *F. oxysporum* is genetically distinct from other *formae speciales* of *F. oxysporum*. Thus, the trefoil-wilt pathogen does not appear to pertain to a previously described *forma specialis* of *F. oxysporum;* because lineages of *F. oxysporum* are highly clonal (the fungus reproduces only asexually), the trefoil-wilt pathogen would be expected to carry sequence identical to at least one race of another *forma specialis* if it pertained to that lineage. The multilocus DNA sequence typing also indicates that the trefoil-wilt pathogen is clonal, and development of a single set of diagnostic PCR primers should be possible.

Trefoil growers throughout the Vermont and New York should benefit from this work. With the movement of people and equipment among regions, the pathogen is likely to be disseminated throughout the region from its current distribution in western New York and the Champlain Valley of New York and Vermont. The knowledge of which other legumes are affected by the pathogen will enable growers to minimize yield losses when making planting decisions for infested fields, and the diagnostic primers, when finished, will permit growers with symptomatic trefoil to obtain rapid, economical and reliable results from diagnostic laboratories. Improved diagnosis should lead to improved yields of trefoil; susceptible trefoil cultivars, which typically exhibit 40 percent stand loss due to the pathogen in the first year of production,[5] are still commonly grown in regions where the pathogen is widespread, and switching to Pardee in infested fields may improve yields.

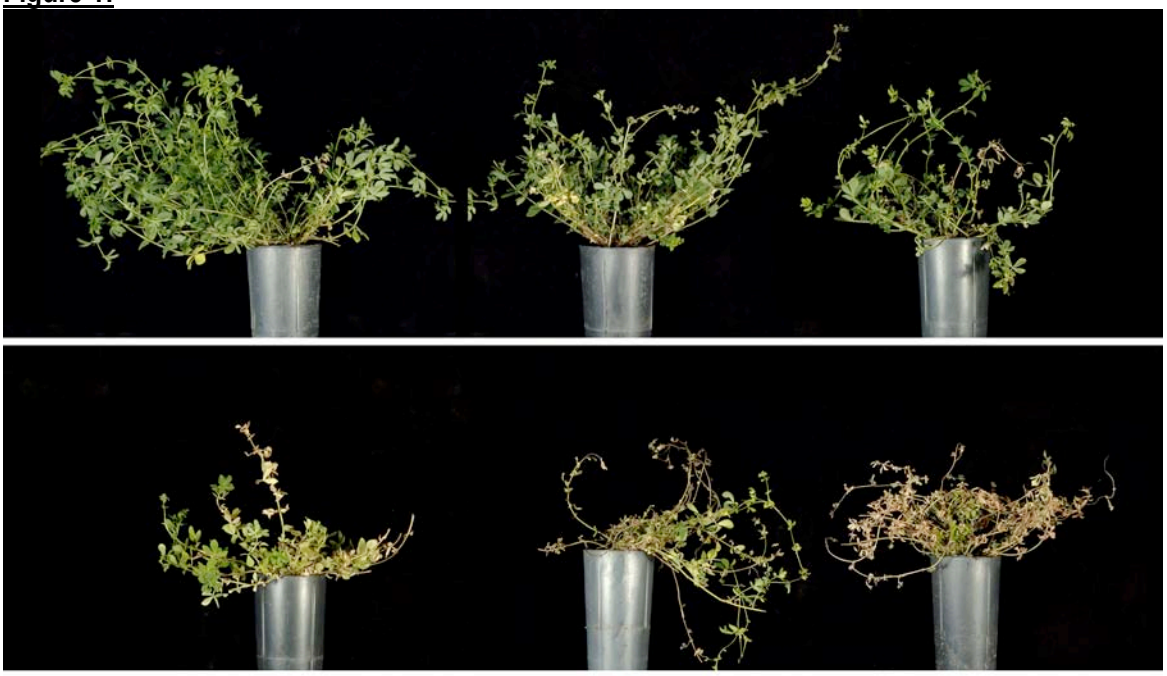
By facilitating the identification of fields where the pathogen is a problem and where a resistant cultivar should be grown and by validating current trefoil breeding strategies, the project contributes to the New York State IPM program priority of developing integrated management strategies for the pathogen. Further, by addressing an important trefoil production problem and identifying an alternative forage legume, alfalfa, not likely to be impacted by the pathogen, the project also contributes to improving the competitiveness of forage legume production relative to alternatives such as grasses and silage corn. Forage legumes, due to their perennial nature and their ability to form symbiotic relationships with nitrogen-fixing *Rhizobium* bacteria, are associated with lower soil erosion and/or lower fertilizer inputs than the grasses and silage corn. The increased competitiveness of forage legumes is thus likely not only to increase the profitability of forage legume production but is likely to also protect water quality in the watersheds where forages are grown.

9. Project locations:

Currently, the trefoil-wilt *F. oxysporum* is only known to occur in New York, Vermont and possibly Ohio, and the research findings will be most useful in these states. However, trefoil is grown across the country; systematic survey efforts for this pathogen have not been conducted in other states, and the results may be applicable in other regions as well.

10. Photographs and figures:

Figure 1:



A typical progression of wilt symptoms on birdsfoot trefoil.

Figure 2:

Trefoil exhibiting typical wilt symptoms

Figure 3:

Roots of trefoil infected with the pathogenic *F. oxysporum*; note that both stele and cortex tissue is necrotic in severe infections (root at farthest right).

Figure 4:

Fusarium oxysporum growing from the ends of surface-sterilized trefoil stem sections on potato dextrose agar

Additional pictures:

M. Wunsch working in the lab, loading a gel as a part of the sequencing efforts.

Table 1:

Incidence of *Fusarium*-associated wilt

by isolate-host combination

	F. oxysporum from trefoil (Vermont)	*F. oxysporum* from trefoil (Vermont)	*F. oxysporum* from trefoil (western NY)	*F. oxysporum* from trefoil (western NY)	*F. oxysporum* from trefoil (western NY)	*F. oxysporum* f. sp. *medicaginis* (Pennsylvania)	*F. oxysporum* from clover (Wisconsin)	*F. oxysporum* f. sp. *pisi*	Control Czapek-Dox broth
Isolate, year collected	**VT 3a, 2004**	**VT 7-1, 2004**	**MK 6-1, 2004**	**Fo069, 1994**	**Fo012-11, 1991**	**Fom004**	**Fo062**	**Fopi001**	
Trefoil, cv. Georgia-1 *(Lotus corniculatus)*	**16**^{bcd}	**19**^{bc}	**21**^{b}	**21**^{b}	**23**^{ab}	0^{e}	0^{e}	1^{e}	0^{e}
Alfalfa, cv. MNGN-1 *(Medicago sativa)*	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	**35**^{a}	0^{e}	0^{e}	1^{e}
Red Clover, cv. Chesapeake *(Trifolium pratense)*	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	7^{bcde}	0^{e}	0^{e}
Pea, cv. M410 *(Pisum sativum)*	1^{e}	3de	7^{bcde}	4^{cde}	4^{cde}	1^{e}	4^{cde}	**35**^{a}	0^{e}
Bean, cv. U.I. 114 *(Phaseolus vulgaris)*	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}
Soybean, cv. Essex *(Glycine max)*	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}

- **Numbers represent the number of plants out of the 24 or 36 tested exhibiting wilt symptoms caused by *Fusarium* sp.**
- **36 plants were tested in every isolate x host combination** except for the isolate x host combination "Vt3a x soybean", in which only 24 plants were tested
- Plants were only scored "positive" for vascular wilt if Fusarium sp. grew from the ends of wilted stem sections that were surface sterilized and plated on 1/8-strength potato dextrose agar,
- **Numbers in bold are significantly different than zero** (P<0.025)
- **Different superscript letters indicate significant difference** (P<0.025)
- Plants were grown individually in separate pots
- The data represent the pooled results from three temporally spaced greenhouse experiments; data were pooled after a chi-square test for heterogeneity resulted in a failure to reject the null hypothesis that the results from the three experiments were homogeneous (P=0.9999).

Table 2:

Root Necrosis

by isolate-host combination

	F. oxysporum from trefoil (Vermont)	*F. oxysporum* from trefoil (Vermont)	*F. oxysporum* from trefoil (western NY)	*F. oxysporum* from trefoil (western NY)	*F. oxysporum* from trefoil (western NY)	*F. oxysporum* f. sp. *medicaginis* (Pennsylvania)	*F. oxysporum* from clover (Wisconsin)	*F. oxysporum* f. sp. *pisi*	Control Czapek-Dox broth
Isolate, year collected	**VT 3a, 2004**	**VT 7-1, 2004**	**MK 6-1, 2004**	**Fo069, 1994**	**Fo012-11, 1991**	**Fom004**	**Fo062**	**Fopi001**	
Trefoil, cv. Georgia-1 *(Lotus corniculatus)*	**2.39**	**2.64**	**3.00**	**2.50**	**2.81**	0.17	0.06	0.56	0.17
Alfalfa, cv. MNGN-1 *(Medicago sativa)*	0.08	0.17	0.39	0.19	0	**4.78**	0.11	0.06	0.19
Red Clover, cv. Chesapeake *(Trifolium pratense)*	0.33	0.61	0.97	0.42	0.47	1.97	**2.53**	0.94	0.14
Pea, cv. M410 *(Pisum sativum)*	0.47	1.08	1.31	1.22	0.97	1.08	0.86	**4.67**	0.11
Bean, cv. U.I. 114 *(Phaseolus vulgaris)*	0.58	0.47	0.61	0.56	0.19	0.53	0.58	0.67	0.06
Soybean, cv. Essex *(Glycine max)*	0.04	0.08	0.03	0.03	0.06	0.03	0.06	0	0.06

- **Numbers represent the mean root necrosis ratings of the 24 or 36 plants tested in each isolate x host combination**
- **36 plants were tested in every isolate x host combination** except for the isolate x host combination "Vt3a x soybean", in which only 24 plants were tested
- **Numbers in bold represent host-isolate combinations in which a high frequency of severe root necrosis was observed.**
- **The following scale was used to rate root necrosis:**
  - 0 = no discoloration of root tissues
  - 1 = small dark strands in the stele
  - 2 = small dark arcs or rings in the stele cross-section
  - 3 = stele mostly dark
  - 4 = entire stele dark, part of cortex dark
  - 5 = entire stele dark, most or all of cortex dark
- Plants were grown individually in separate pots.
- The data represent the pooled results from three temporally spaced greenhouse experiments; data were pooled after a chi-square test for heterogeneity resulted in a failure to reject the null hypothesis that the results from the three experiments were homogeneous (P=0.6148).

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